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Differential Effects of Three Canonical *Toxoplasma* Strains on Gene Expression in Human Neuroepithelial Cells▽†

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Strain type is one of the key factors suspected to play a role in determining the outcome of *Toxoplasma* infection. In this study, we examined the transcriptional profile of human neuroepithelioma cells in response to representative strains of *Toxoplasma* by using microarray analysis to characterize the strain-specific host cell response. The study of neural cells is of interest in light of the ability of *Toxoplasma* to infect the brain and to establish persistent infection within the central nervous system. We found that the extents of the expression changes varied considerably among the three strains. Neuroepithelial cells infected with *Toxoplasma* type I exhibited the highest level of differential gene expression, whereas type II-infected cells had a substantially smaller number of genes which were differentially expressed. Cells infected with type III exhibited intermediate effects on gene expression. The three strains also differed in the individual genes and gene pathways which were altered following cellular infection. For example, gene ontology (GO) analysis indicated that type I infection largely affects genes related to the central nervous system, while type III infection largely alters genes which affect nucleotide metabolism; type II infection does not alter the expression of a clearly defined set of genes. Moreover, Ingenuity Pathways Analysis (IPA) suggests that the three lineages differ in the ability to manipulate their host; e.g., they employ different strategies to avoid, deflect, or subvert host defense mechanisms. These observed differences may explain some of the variation in the neurobiological effects of different strains of *Toxoplasma* on infected individuals.

Toxoplasma gondii is a protozoan parasite that infects approximately 25% of the world's human population (26). Most *Toxoplasma* isolates that have been identified in Europe and North America belong to three distinct clonal lineages (23), referred to as types I, II, and III. Despite having >98% genetic identity, the three types display a number of phenotypic differences in mice, including virulence, persistence, migratory capacity, attraction of different cell types, and induction of cytokine expression (43). These phenotypic differences are related to primary amino acid sequence differences and/or quantitative differences in gene expression (44). Recent studies indicate that such differences might also occur in human infections with *Toxoplasma*. For example, type I strains have been shown to be more common in ocular toxoplasmosis (17). Infection with type I strains in mothers has also been reported to be associated with an increased risk of psychosis in the offspring (57). Type II strains cause the majority (70 to 80%) of human cases of toxoplasmosis reported previously in North America and Europe (primarily France) (22). Type III strains are found largely in animals and only occasionally have been described in association with human toxoplasmosis, for reasons that are unknown (22). Those studies suggested that the genetic makeup of *Toxoplasma* plays an important role in the outcome of toxoplasmosis, although host genetic factors are also involved (50).

It is well-known that infection with *T. gondii* can affect the cognition and behavior of rodents. Infected rodents show impaired learning and memory as well as increased activity (4). One of the most remarkable changes is that *T. gondii* infection can convert the rodents' natural aversion to feline odors into attraction (53), presumably in order to alter the behavior of their hosts to benefit the parasites' sexual cycle (55). Several studies have also investigated the effects of *T. gondii* on human personality characteristics and behavior and found that *Toxoplasma*-infected individuals have a significantly increased risk of traffic accidents compared to noninfected ones (12). It is clear that congenital infections with *T. gondii*, especially following primary infection early in pregnancy, can produce intracranial calcifications, mental retardation, deafness, seizures, and retinal damage. Long-term effects of congenital infection that occur late in pregnancy include cognitive symptoms such as lower IQ and microcephaly (47).

Strain-specific differences of *Toxoplasma* in the modulation of host cell transcription have been identified previously. Studies in human fibroblasts infected with the three types have documented the differential expression levels of more than 88 genes that were regulated in a strain-specific manner (44). Previous studies have also documented cell-specific responses to *Toxoplasma* type I in dendritic cells, macrophages, and retinal vascular endothelial cells (8, 30). The brain represents an important target organ for *Toxoplasma* in terms of the establishment of persistence and the alteration of host behavior. Although a large body of work has been carried out to identify transcriptional differences among different cells infected with *Toxoplasma*, similar studies on neural cells have not been re-

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ported. We thus examined gene expression in neuroepithelial cells following infection with representative strains of the three *Toxoplasma* types. We found that the different *Toxoplasma* strains elicit marked differences in gene expression in infected cells. These differences are both quantitative and qualitative and involve a wide range of biological functions.

MATERIALS AND METHODS

Parasites and cells. The human neuroepithelioma cell line SK-N-MC (ATCC HTB-10) was used for all assays and was propagated as described previously (13). This cell line was chosen for study in light of the ability to propagate it under defined and reproducible conditions and to generate sufficient amounts of standardized target material (2). The following *T. gondii* strains representing the three major clonal types were used in this study: 2F (type I, ATCC 50839), which constitutively expresses cytoplasmic β-galactosidase and is derived from the RH strain, PRU (type II, provided by V. B. Carruthers, University of Michigan School of Medicine), and CTG (type III, ATCC 50842). *Toxoplasma* strains were maintained by passage in SK-N-MC monolayers cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 3% fetal bovine serum (FBS).

Infection or mock infection of SK-N-MC cells. SK-N-MC cells were plated in 6-well trays. Upon reaching a confluence of 30 to 50%, cell cultures were synchronized by serum deprivation (0.5% FBS) for 2 days. Parasites were released from intracellular vacuoles by syringe lysis, filter purified (13), and then resuspended in Endo buffer (44.7 mM K_2SO_4, 10 mM $MgSO_4$, 106 mM sucrose, 5 mM glucose, 20 mM Tris-H_2SO_4, 3.5 mg/ml BSA, pH 8.2), a potassium buffer used in the synchronization of tachyzoite invasion (27). Briefly, at the time of infection, the synchronized cells were inoculated with tachyzoites at a multiplicity of infection (MOI) of 3 or with Endo buffer alone (mock-infected controls). After parasites were added, the 6-well tray was centrifuged at 500 × *g* for 2 min and permitted to settle for 20 min at 37°C. Endo buffer was then aspirated, replaced with 3% serum medium, and incubated for 20 h. Total RNA was harvested using RNeasy reagent (Qiagen, Valencia, CA). Infections and controls (no tachyzoites) were performed in duplicate, and experiments for each strain were carried out on three separate occasions in order to include both technical replicates and biological replicates.

Microarray analysis. RNA transcript levels were quantified by microarray analyses according to previously published procedures (56). Briefly, RNAs were amplified into cRNAs and biotinylated by *in vitro* transcription with Affymetrix reagents, using the Whole Transcript Sense Target Labeling protocol as described in the Affymetrix manual. Biotinylated cRNAs were purified, fragmented, and subsequently hybridized to Affymetrix GeneChip human exon 1.0 ST arrays.

Data normalization and statistical analysis. Gene expression analysis was performed using an Affymetrix GeneChip human exon 1.0 ST array. Data analysis began with raw GeneChip data in the form of Affymetrix CEL files, which were produced and assigned quality control scores by GeneChip Command Console software. These data were extracted and normalized with Partek Genomics Suite software using the Robust Multichip Analysis (RMA) algorithm (24). To ensure exploration of the broadest range of gene transcripts, we imported all Affymetrix "extended probes," yielding transcripts of which 76,206 currently have annotation with 43,004 at the gene or mRNA level.

Transcription analysis first summarized each transcript's exons into a single expression value for transcript-level comparisons to detect differential gene expression between samples under differing conditions. A two-way analysis of variance (ANOVA) was used to detect genes with statistically significant expression levels between *Toxoplasma* strains and their corresponding sets of mock-infected cells. Due to the exploratory nature of the study and the fact that the brain normally shows small changes in gene expression (18, 37), genes whose transcripts were judged to be differentially expressed, meaning they had to have an ANOVA *P* value of ≤0.01 and a change in expression of at least 1.2-fold in either direction, were selected as candidates for further functional analyses (54, 58).

Data mining. Gene Ontology (GO) analysis was conducted for differentially expressed genes using the Spotfire analytic platform. Selected genes were compared against the universe of unselected genes at each level of annotation, and for each, a *P* value was calculated according to a hypergeometric distribution that represents the probability that the gene functions are randomly distributed between groups (51). Network, function, and pathway analyses were generated using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Mountain View, CA), which assists with microarray data interpretation via grouping differentially expressed genes into known functions, pathways, and networks.

TABLE 1. Numbers of differentially expressed RefSeq genes, altered canonical pathways, and altered networks

Infection	No. of RefSeq genes[a]		No. of altered canonical pathways[b]	No. of altered networks[c]
	Upregulated	Downregulated		
Type I	396	726	34	25
Type II	54	24	4	2
Type III	197	147	16	15

[a] $P < 0.01$; >1.2-fold change in expression.
[b] $P < 0.05$.
[c] IPA score, >3.

Real-time PCR. To validate differentially expressed genes, the same RNA used for microarray analysis was used for real-time quantitative PCR. The RNA was first treated with Turbo DNA-free DNase (Applied Biosystems, Foster City, CA) to remove trace amounts of genomic DNA. Reverse transcription was performed using MultiScribe reverse transcriptase and random primers as recommended by the manufacturer (Applied Biosystems, Foster City, CA). Real-time analysis was carried out using FAM master mix (Applied Biosystems, Foster City, CA) with 30 ng of a cDNA template in a 10-μl reaction volume, and all real-time reactions were carried out in triplicate. The PCRs were analyzed on an ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA). The fold changes in expression between groups were evaluated using relative quantization (threshold cycle [$\Delta\Delta C_T$] method) with β-actin endogenous controls (demonstrating low variation from microarray analysis). Real-time results were analyzed using SDS software (version 2.2.1; Applied Biosystems), with automatic computation of baseline and threshold fluorescence levels. The genes chosen for validation were selected based on their involvement in neurotransmitter processes and on the strength of the findings from the microarray analysis.

Microarray data accession number. All microarray data obtained in this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) public repository, and they are accessible through GEO accession number GSE22986.

RESULTS

Gene expression profiling in SK-N-MC cells. After 20 h, exposure of SK-N-MC cells to 3 *T. gondii* strains resulted in differing patterns of gene expression, both quantitatively and qualitatively (see Data Set S1 in the supplemental material). Infection with *T. gondii* type I caused a significant change of expression in ~3.3% of transcripts (1,423 out of 43,004; $P < 0.01$; >1.2-fold change) on the array compared to no infection. In contrast, only 0.4% (162 out of 43,004) and 1.1% (478 out of 43,004) of transcripts were altered by type II and type III infections, respectively. The proportion of detected transcripts was substantially higher among RefSeq genes than among non-RefSeq genes, reflecting the greater degree of knowledge and certainty about the existence of RefSeq transcripts. In type I strain-infected cells, we observed 1,122 RefSeq genes with significant transcriptional changes. Among them, 726 were downregulated and 396 were upregulated. In type II strain-infected cells, 78 RefSeq genes demonstrated significant transcriptional changes, including 24 downregulated and 54 upregulated genes. There were 344 RefSeq genes with significant transcriptional changes in type III strain-infected cells, with 147 downregulated and 197 upregulated genes (Table 1). Interestingly, more genes were repressed than activated in type I strain-infected cells, in contrast with that seen in cells infected by the type II or type III strain.

There were 119 RefSeq genes that overlapped between type I and type III strain infections, while only 14 genes overlapped

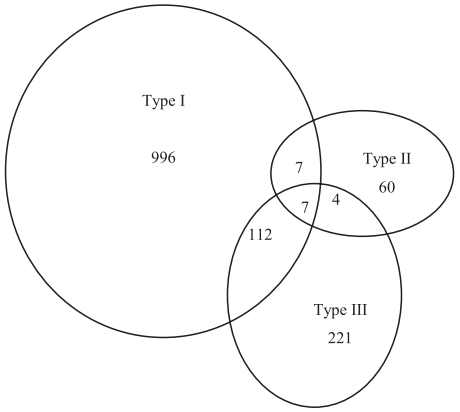

FIG. 1. Venn analysis of the overlap of deregulated RefSeq genes in SK-N-MC cells due to different *Toxoplasma* infections. In the SK-N-MC cells infected by *Toxoplasma* type I compared to controls, type II compared to controls, and type III compared to controls, respectively, 1,122, 78, and 344 RefSeq gene transcripts were differentially expressed. The intersections of the circles indicate the number of genes in common between contrasts.

between type I and type II strain infections and only 11 genes between type II and type III strain infections (Fig. 1). This finding is consistent with previous results which showed that type I and type III strains share more identical alleles at many loci (31). In addition, there were 7 RefSeq genes (*VIPR2*, *LOC401620*, *TAS2R39*, *LOC645317*, *CSN1S2A*, *ALG1*, and *KRT37*), all of which were upregulated, significantly affected by all three strains (Fig. 1).

Identification of biological processes modulated by *Toxoplasma*. Differentially expressed genes (DEGs) between infected and mock-infected cells were analyzed using Gene Ontology (GO; www.geneontology.org) to identify altered biological processes. In type I strain-infected cells, GO analysis revealed a marked effect in processes related to reproduction, response to stimulus, motility, metabolism, homeostasis, the central nervous system, inflammation, apoptosis, behavior, and transport. In type II strain-infected cells, overrepresented ontologies were found to be related to circadian rhythm, growth, and signaling. Analysis in type III strain-infected cells revealed an overrepresentation of processes related to nucleic acid metabolism, protein targeting, transport, cellular response to stimulus, protein localization, gene expression, localization, metabolism, nuclear export, and signaling (Table 2; see also Data Set S7 in the supplemental material).

Nervous system development. In type I strain-infected cells, the most remarkable brain-related finding was the large number of enriched GO categories ($n = 17$) related to nervous system development (Fig. 2; see also Data Set S2 in the supplemental material). For example, the development of the limbic system, subpallium, striatum, and hippocampus are child nodes related to nervous system development. A total of 76 DEGs corresponded to this category, where a large proportion of these 76 genes (28 out of 76 or 36.8%) corresponded to genes implicated in the central nervous system. More specifically, it was remarkable that 82.1% of genes (23 out of 28) were implicated in brain development. As seen in Fig. 2, a clear pattern of gene dysregulation was observed, with a majority of

TABLE 2. Enriched top biological processes from Gene Ontology analysis using differentially expressed genes

Infection and category	No. of genes belonging to category	% of total GO annotated genes[a]	*P* value
Type I			
Regulation of microtubule-based movement	4	100	7.09E−06
Female pregnancy	17	16.0	2.90E−05
Anatomical structure development	166	6.60	2.67E−04
Regulation of action potential	12	16.7	2.94E−04
Striatum development	4	50.0	4.20E−04
Body morphogenesis	5	33.0	4.94E−04
One-carbon metabolic process	16	12.7	7.75E−04
Muscle system process	23	10.6	9.27E−04
Nervous system development	76	7.40	1.01E−03
Response to endogenous stimulus	36	8.89	1.08E−03
Generation of precursor metabolites and energy	31	9.30	1.18E−03
Negative regulation of immune system process	11	13.8	2.64E−03
Hippocampus development	5	23.8	3.71E−03
Limbic system development	6	19.4	4.54E−03
Head development	4	28.6	4.68E−03
Regulation of inflammatory response	10	13.3	5.08E−03
Regulation of neuronal synaptic plasticity	6	18.8	5.35E−03
Regulation of apoptosis	59	7.20	6.24E−03
Circulatory system process	21	9.30	6.48E−03
Central nervous system segmentation	2	66.6	7.74E−03
Rhythmic behavior	4	25.0	7.84E−03
Blastocyst development	6	17.1	8.41E−03
Brain development	23	8.80	8.91E−03
Memory	6	16.7	9.66E−03
Type II			
Nuclear body organization	1	50.0	4.41E−03
Positive regulation of circadian sleep/wake cycle and rapid eye movement sleep	1	33.3	6.60E−03
Positive regulation of growth hormone secretion	1	20.0	1.10E−02
Positive regulation of insulin-like growth factor receptor signaling pathway	1	20.0	1.10E−02
B cell receptor signaling pathway	1	11.1	1.97E−02
Nerve growth factor receptor signaling pathway	1	11.1	1.97E−02
Positive regulation of erythrocyte differentiation	1	10.0	2.04E−02
Positive regulation of cellular component movement	2	2.00	2.19E−02
Reproductive cellular process	1	7.10	2.76E−02
Acrosome reaction	1	7.70	2.83E−02
G protein-coupled receptor protein signaling pathway	6	0.50	3.46E−02
Negative regulation of cell proliferation	3	0.80	4.04E−02
Type III			
ncRNA processing	16	8.30	1.41E−07
ncRNA metabolic process	15	6.40	9.16E−06
Ribosome biogenesis	11	8.30	1.25E−05
Primary metabolic process	150	2.00	8.74E−04
Gene expression	83	2.20	1.25E−03
Cellular response to extracellular stimulus	5	8.20	3.39E−03
Nuclear export	5	7.20	5.76E−03
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	81	2.10	6.70E−03
Ionotropic glutamate receptor signaling pathway	2	25.0	7.21E−03
Protein targeting	10	4.00	8.87E−03
Mitochondrial transport	5	6.60	9.10E−03
Regulation of neuronal synaptic plasticity	3	9.40	1.58E−02

[a] This value represents the number of differentially expressed genes that map to a given category divided by the total number of genes in that category.

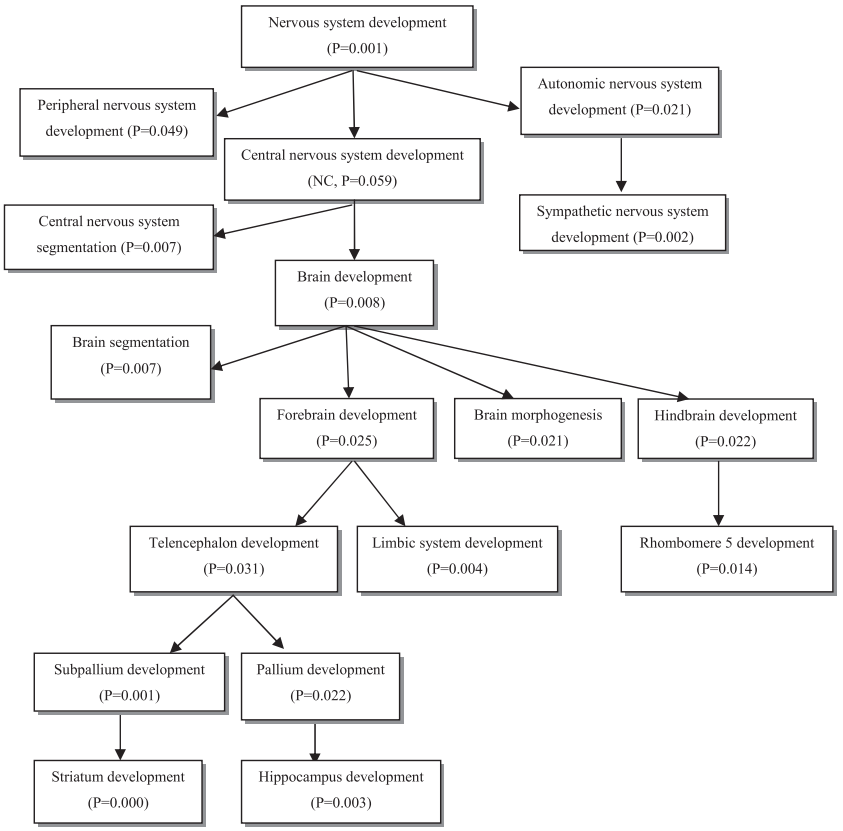

FIG. 2. Transcriptional changes of biological processes involved in nervous system development affected by type I strain infection. Shown are the biological processes with significant *P* value changes in SK-N-MC cells altered by *Toxoplasma* type I infection. NC, no significant change.

forebrain, hindbrain, and brain morphogenesis genes being downregulated. For instance, in the forebrain, 11 out of 13 (84.6%) DEGs were downregulated (*APAF1*, *NDE1*, *BBS2*, *RAX*, *IFT88*, *NOTCH3*, *ATP7A*, *BBS4*, *MKKS*, *APLP1*, and *DRD1*). Correspondingly, in the hindbrain, 5 out of 7 (71.4%) DEGs were downregulated (*MAFB*, *IGF1*, *MYO16*, *ATP7A*, and *GAS1*).

Transmission of nerve impulse. Transmission of nerve impulse was another process determined to be most enriched in SK-N-MC cells infected by the type I strain. A total of 8 GO categories, including 31 genes implicated in the transmission of nerve impulse, were enriched (Fig. 3; see also Data Set S3 in the supplemental material). A good portion of these genes ($n = 10$) corresponded to the regulation of action potential in

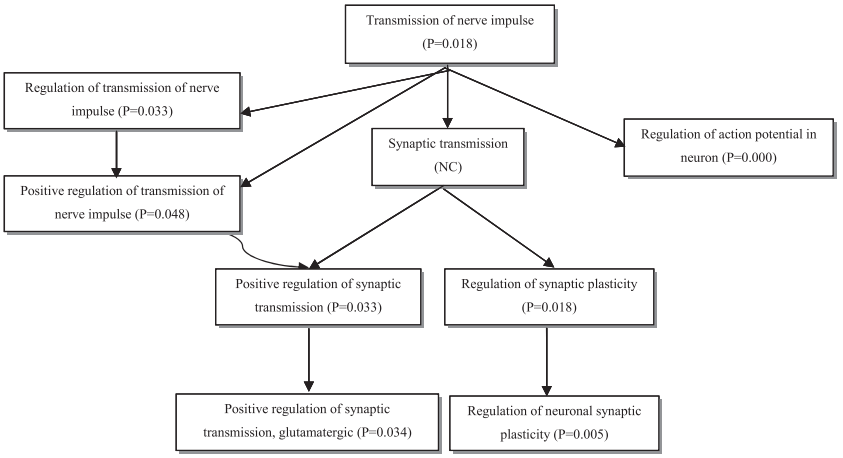

FIG. 3. Transcriptional changes of biological processes involved in nerve impulse transmission affected by type I strain infection. Shown are the biological processes with significant *P* value changes in SK-N-MC cells altered by *Toxoplasma* type I infection. NC, no significant change.

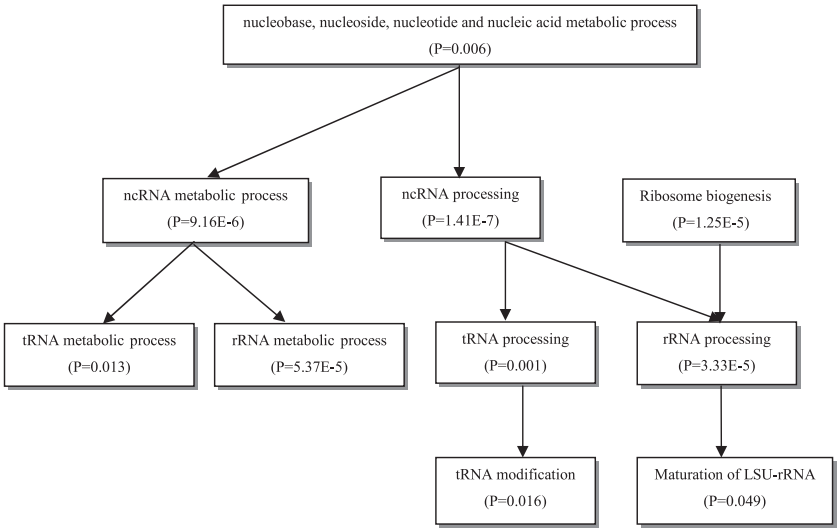

FIG. 4. Transcriptional changes of biological processes involved in the nucleotide metabolic process affected by type III strain infection. Shown are the biological processes with significant *P* value changes in SK-N-MC cells altered by *Toxoplasma* type III infection. LSU, large subunit.

neurons, with the majority ($n = 7$) being downregulated (*CLDN1*, *ILK*, *SCN2A*, *ERBB2*, *DRD1*, *KCNMB4*, and *SCN8A*). In addition, we found that most of the rest of the genes corresponding to synaptic transmission, especially in the regulation of synaptic plasticity, were upregulated. For example, in the regulation of synaptic plasticity, 67% (6 out of 9) of DEGs were clearly upregulated, which included several postsynaptic proteins (*EGR1* and *EGR2*) and regulation proteins (*ARC*, *PTGS2*, *ADORA1*, and *GRIN2A*).

Metabolic processes. This GO analysis revealed a marked effect on nucleoside, nucleotide, and nucleic acid metabolism in SK-N-MC cells infected by the type III strain, with a majority of genes being upregulated (see Data Set S4 in the supplemental material). A total of 10 GO categories exhibit signs of enrichment in this process (Fig. 4), mostly related to the metabolism and processing of noncoding RNAs (ncRNA). Genes within the ncRNA categories correspond to a number of RNA processing and modification proteins (*RRP9*, *BOP1*, *RRP1*, *TSR2*, *TRMT1*, *NOL6*, *EXOSC6*, *IMP4*, *TRMU*, *DDX56*, *DUS3L*, *TRMT61A*, and *METTL1*) and several transcription regulation proteins (*TARBP2*, *PWP2*, and *PUS1*).

Compared with infection by the other strains, type II strain infection yielded a small number of biological process alterations, and those changes that did occur were almost all of lower magnitude (Table 2). Therefore, we found no clear trends for any of these processes being involved in particular categories.

Gene networks among DEGs during *Toxoplasma* infection. We performed Ingenuity Pathways Analysis (IPA) on each data set of DEGs to generate networks which indicate the molecular relationships between genes/gene products (see Data Set S5 in the supplemental material). IPA computes a score for each network according to the fit of the user's set of significant genes. In type I strain-infected cells, IPA revealed 25 networks with highly significant scores from 15 to 46. In type II strain-infected cells, IPA revealed only 2 networks with scores over 3. In type III strain-infected cells, there were 15 networks with scores over 3.

Several of these networks revealed molecular functions that could be coupled to cell communication. We combined the top 5 networks (1, 3, 16, 17, 20) from the type I strain infection, whose function was related to cell signaling, and used IPA to investigate molecular interactions between these molecules (Fig. 5). It appears that v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), extracellular signal-regulated kinase 1/2 (ERK1/2), transcription regulator TP53 (tumor protein p53), gamma interferon (IFN-γ), and interleukin 4 (IL-4) are connected to a majority of molecules within this mergence. Cells infected by the type II strain have only one signaling-related network, in which proinflammatory cytokine tumor necrosis factor (TNF), ERK1/2, and IL-4 appear to be connected to the majority of molecules, although none of them showed a significant change in expression (see Fig. S1 in the supplemental material). However, this pattern suggests that most genes differentially expressed by type II infection could be coupled to these molecules, either directly or through mediators like TNF-AIP6. Similarly, by combining the top 4 networks from type III strain infection (2, 4, 5, and 6), whose function was related to cell signaling, TNF and ERK1/2 were connected to a majority of molecules (see Fig. S2 in the supplemental material).

Canonical pathway analysis using IPA. The mapped DEGs within each data set were subsequently analyzed for canonical pathways to explore the key biological pathways modulated by different *Toxoplasma* infections (see Data Set S6 in the supplemental material). In type I strain-infected cells, we uncovered 34 significantly enriched canonical pathways ($P < 0.05$), the majority of which were signaling pathways. In type II strain-infected cells, only 4 common pathways were modulated, all of which were signaling pathways. In type III strain-infected cells, we identified 16 common pathways. It was striking that both metabolic pathways and signaling pathways were equally affected. The top enriched canonical signaling and metabolic

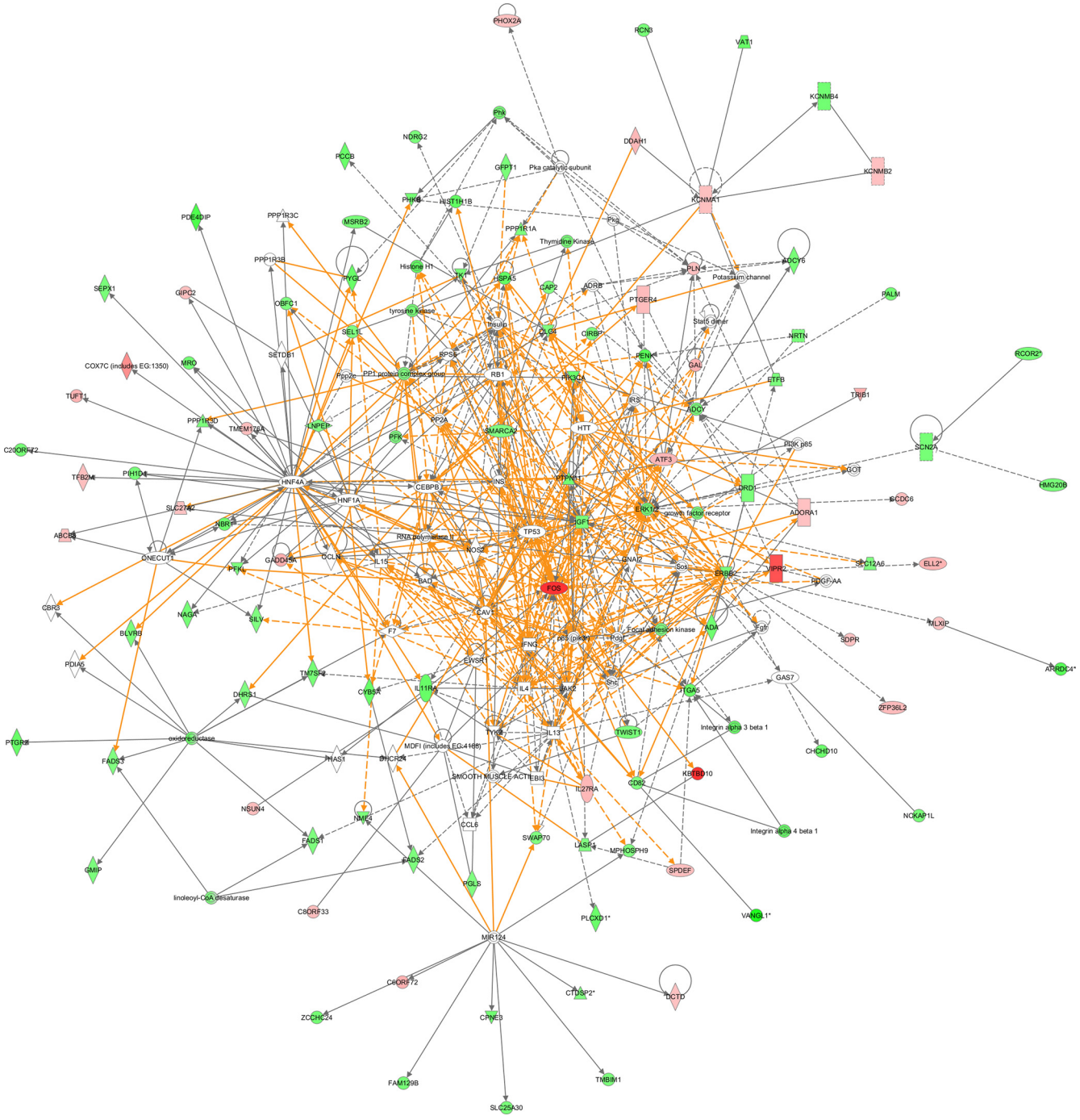

FIG. 5. Ingenuity signaling-related networks depicting interactions among molecules. Merged network based on the top 5 signaling-related networks (107 differentially expressed genes [DEGs] and 66 interconnecting molecules) altered by *Toxoplasma* type I infection. Red denotes upregulation, and green denotes downregulation of the gene. The intensity of the gene color (red or green) indicates the degree of up- or downregulation. White indicates interconnecting molecules. Lines between molecules indicate a direct molecular connection between molecules. Gold lines indicate connectivity between the merged networks. Asterisks indicate duplicates. The gene shapes denote the functional class of the gene product. CoA, coenzyme A; ORF, open reading frame. The networks were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems).

pathways ($P < 0.01$) are reported in Table 3. It is notable that in most cases fewer than 10% of the genes in known pathways were detected. We currently do not know the extent to which a significant P value will correspond to a biologically relevant effect. There is no consensus definition of biological significance. However, the statistically significant altered canonical pathway may provide a rational basis for further studies directed at the assessment of biological activity.

TABLE 3. Top canonical pathways from Ingenuity Pathways Analysis using differentially expressed genes

Infection and canonical pathway	*P* value[a]	Ratio[b]	Genes[c]	Function(s)
Type I				
Signaling pathways				
IL-8 signaling	2.71E−03	0.085	*PLCB2*, ***RND3***, *VCAM1*, *PLD3*, *PTK2B*, *RHOC*, *RRAS*, *PGF*, ***NFKB1***, *PLD1*, *PIK3CA*, *MAPK3*, ***PTGS2***, ***GNG12***, *FNBP1*, *IRAK4*	Plays a central role in angiogenesis, tumor growth, and inflammation
Cholecystokinin/gastrin-mediated signaling	3.70E−03	0.106	*MAP2K6*, ***FOS***, *PLCB2*, ***SRF***, ***RND3***, *PTK2B*, *RRAS*, *RHOC*, *FNBP1*, *PLCB3*, *MAPK3*	Stimulates acid secretion from the parietal cell and an important growth/differentiation factor for the entire gastrointestinal tract
IGF-1 signaling	7.66E−03	0.098	***FOS***, *PIK3CA*, ***CTGF***, *IGF1*, *PTPN11*, *RRAS*, *MAPK3*, ***SRF***, ***IGFBP3***, ***CYR61***	Promotes cell proliferation, growth, and survival
Metabolic pathways				
Pyrimidine metabolism	1.94E−03	0.065	***DPYSL2***, *RP2*, *NME4*, *PRIM1*, *POLI*, ***DCTD***, ***PUS1***, ***TXNRD1***, ***POLR1A***, ***POLR1B***, *RRM2B*, ***POLR1C***, ***NT5E***, *TK1*, ***POLE3***	Nucleotide metabolism; provides the sources of energy that drive most reactions
Ascorbate and aldarate	5.79E−03	0.058	*ALDH1B1*, *CYP24A1*, *FADS3*, *ALDH9A1*, *ALDH7A1*	Carbohydrate metabolism
Type II				
Prolactin signaling	9.34E−03	0.026	***NMI***, ***PDK1***	Reproduction and lactation, growth and development, endocrinology and metabolism, brain and behavior, and immune modulation and osmoregulation
Type III				
Signaling pathways				
CD27 signaling in lymphocytes	7.19E−04	0.088	*MAP2K6*, ***SIVA1***, *APAF1*, ***BID***, ***NFKBIB***	Important for cell growth and survival
PKR in interferon induction and antiviral response	3.01E−03	0.087	*MAP2K6*, *APAF1*, ***BID***, ***NFKBIB***	Triggers antiviral effects mainly by apoptosis of the host cell or inhibition of translation
Metabolic pathways				
Histidine metabolism	7.19E−04	0.042	*HNMT*, ***METTL1***, ***MEPCE***, ***SMOX***, ***METTL7B***	Amino acid metabolism
Pyrimidine metabolism	9.68E−04	0.035	***POLR3K***, ***POLR1C***, ***PUS1***, ***POLR1B***, ***POLR3E***, ***POLR1E***, ***POLE3***, ***POLR1A***	Nucleotide metabolism; provides the sources of energy that drive most reactions
Tyrosine metabolism	2.52E−03	0.025	***METTL1***, ***MEPCE***, ***SMOX***, ***METTL7B***, *LRTOMT*	Amino acid metabolism
Purine metabolism	9.95E−03	0.023	***POLE3***, ***POLR1A***, ***POLR1E***, ***POLR3K***, ***POLR1C***, ***ATP6V0B***, ***POLR1B***, ***POLR3E***, ***PFAS***, *HLTF*	Nucleotide metabolism

[a] *P* values denote the significance of the enrichment of a function within the differentially expressed genes (DEGs).
[b] The number of genes from the data set that map to the pathway (DEGs) divided by the total number of genes in a given pathway.
[c] Names of DEGs in the pathway. Upregulated genes appear in bold, and downregulated genes are in roman.

Most of the pathways altered by type I strain infection appear to be inhibited, since the majority of DEGs within these pathways were downregulated (see Data Set S6 in the supplemental material). Among signaling pathways, those data revealed that the inflammatory response was limited (e.g., apparent inhibition of IL-8 signaling can decrease inflammation, and inhibition of HMGB1 [high-mobility group box 1] signaling results in the blockage of proinflammatory cytokines and chemokines). Detailed analyses of metabolic pathways indicated an overall decrease in carbohydrate (ascorbate and aldarate metabolism and propanoate metabolism) and lipid (sphingolipid metabolism) metabolism. While pyrimidine metabolism was the most affected metabolic pathway, the detailed analysis did not indicate induction or inhibition. Moreover, those signaling pathways revealed a unique landscape where several disease-specific pathways have been altered, such as glioma invasiveness signaling, estrogen-dependent breast cancer signaling, and acute myeloid leukemia signaling. The significance of these pathways is currently unknown.

All 4 canonical pathways altered by type II strain infection involved signaling (see Data Set S6), and genes within these pathways were generally upregulated. Among them, the prolactin and hepatocyte growth factor (HGF) signaling pathways were the most enriched.

Among the top canonical pathways altered by type III strain infection, it was striking that metabolic pathways ($n = 4$) were the most affected and few ($n = 2$) were signaling pathways (Table 3). The two signaling pathways (CD27 signaling in lymphocytes and protein kinase receptor [PKR] in interferon induction and antiviral response) were related to immune response. The 4 metabolic pathways were related to amino acid metabolism (histidine metabolism and tyrosine metabolism) and nucleotide metabolism (pyrimidine metabolism and purine metabolism), with the majority of genes being upregulated.

Real-time PCR validation. We employed real-time PCR to confirm the expression of four upregulated (*VIPR2*, *FOS*, *KBTBD10*, and *HTR1D*) and two downregulated (*DRD1* and *RIT1*) genes in type I strain-infected samples, three upregulated (*VIPR2*, *TAS2R39*, and *GHRH*) genes in type II strain-

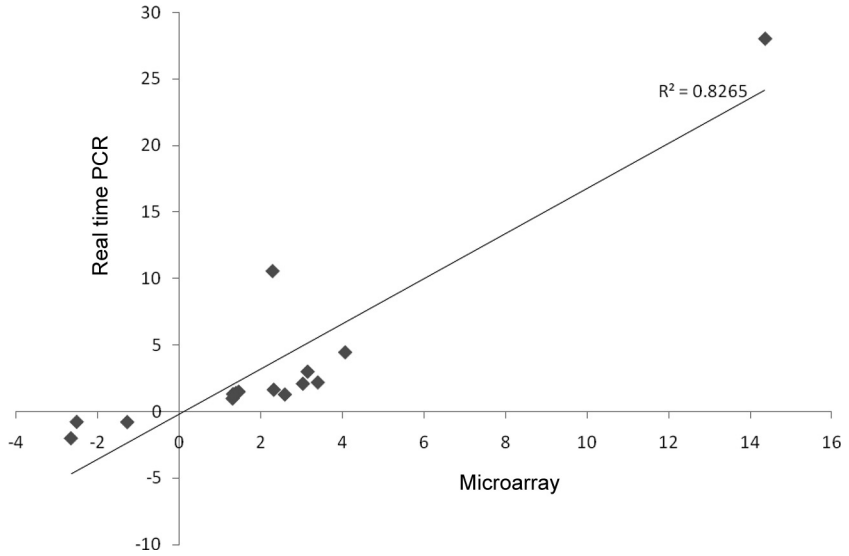

FIG. 6. Correlation between microarray and real-time PCR data. The scatter plot compares ratios of mean expression levels of infected cells to those of controls for 15 genes. Each point represents the infected cell/control ratio from microarray (*x* axis) and real-time PCR (*y* axis) analyses. The Pearson correlation coefficient is indicated on the scatter plot.

infected samples, and five upregulated (*VIPR2*, *TAS2R39*, *GRIN2A*, *SAFB*, and *ADRB3*) and one downregulated (*RAB43*) gene in type III strain-infected samples. The quantitative PCR expression data of these 15 genes showed positive correlation with our microarray expression data ($r = 0.827$) (Fig. 6; see also File S1 in the supplemental material). We also measured the amount of *Toxoplasma* DNA in each group of infected cells to check the replication difference among the infected cells, and no significant differences were found (see File S1).

DISCUSSION

The main goal of the study was to characterize the strain-specific host cell response to the 3 canonical *T. gondii* strains by using human neuroepithelioma cells. Our study showed that the extents of the expression changes varied considerably among the three strains: type I strain-infected cells exhibited the most differential gene expression, whereas type II strain-infected cells exhibited relatively little and type III strain-infected cells exhibited intermediate levels of differential gene expression. There was only a small degree of overlap among the 3 strains in terms of the specific genes that were differentially regulated. These results suggest that the three *Toxoplasma* strains differ in their quantitative and qualitative effects on gene expression in neural cells.

Transcript changes. Neuroepithelial cells infected with *Toxoplasma* type I experienced a wide transcriptional response, encompassing >1,000 genes, while cells infected with type II and type III strains resulted in the alteration of 78 and 344 genes, respectively. This difference occurred despite the high level of genetic similarity among the 3 strains. It is possible that a differential rate of parasite growth could have contributed to some of these differences. However, the effect of differential growth is unlikely to be large in light of the fact that parasite DNA levels measured 20 h following infection of the 3 *Toxoplasma* types did not differ to a statistically significant effect (see File S1 in the supplemental material). This observation is also consistent with previous reports (43, 48). Our findings here are consistent with other studies documenting distinct differences in cellular tropism and virulence among the 3 *Toxoplasma* types (43). Our data are also in agreement with the results of Saeij et al. (44), who found that more than 88 unique genes were regulated in a strain-specific manner using human foreskin fibroblast cells. Differences in the absolute number of genes and the specific genes regulated may be explained by differences in the cell lines, array platforms, and statistical methods that were used in the different studies.

It is of note that we found seven genes (*VIPR2*, *LOC401620*, *TAS2R39*, *LOC645317*, *CSN1S2A*, *ALG1*, and *KRT37*) that were modulated by all three *Toxoplasma* strains in similar manners. It is noteworthy that *ALG1* (asparagine-linked glycosylation 1 homolog) participates in the formation of the lipid-linked precursor oligosaccharides for N glycosylation. *Toxoplasma* has been shown to scavenge N glycosylation intermediates from host cells, presumably to compensate for the rapid evolution of its biosynthetic pathway, which is devoted primarily to modification of proteins with glycosylphosphatidylinositols (15). Moreover, it was recently reported that N glycosylation is essential for successful infection (35). Our finding that the *ALG1* gene is highly upregulated by all three strains is in agreement with those previous findings. In addition, the finding that *VIPR2* is modulated by all strains is quite interesting in terms of the postulated importance of VIP/PACAP in human brain diseases and its role in GABAergic transmission and possible role in schizophrenia (9, 19, 49). The finding that *TAS2R39*, a gene which encodes a taste receptor, is differentially regulated is also of potential interest. In light of the small number of genes that are differentially regulated by all 3 strains, these genes might be of interest in terms of shared pathogenic potential within the central nervous system.

Significant transcript changes specific to type I infection. (i) Nervous system changes. The GO analysis showed alterations in both brain development and nerve impulse transmission in type I-infected cells. Alteration in brain development includes forebrain, hindbrain, brain segmentation, and brain morphogenesis (Fig. 2). The alteration of nerve impulse transmission focuses on the induction of synaptic plasticity and the inhibition of action potential regulation in neurons (Fig. 3). Synaptic plasticity is regarded as the molecular basis of learning and memory (16). Impairments in synaptic transmission and plasticity could disturb the functional coupling between brain areas. Several studies have demonstrated a correlation between synaptic defects observable at the larval neuromuscular junction and impaired learning in *Drosophila* (59). Abnormal sciatic nerve action potential, including amplitude and latency, has been observed in mice chronically infected with *Trypanosoma cruzi*, depending on the parasite strain (52). Consequently, the chronically infected mice present a reduced number of functional axons within the nerve and demyelination of conducting fibers (34). Whether alteration caused by *Toxoplasma* type I has analogous effects awaits future investigation.

In immunocompromised patients, *Toxoplasma* infection most often involves the nervous system, with diffuse encephalopathy, meningoencephalitis, or cerebral mass lesions (14). In immunocompetent individuals, *Toxoplasma* infections may have significant effects on host behavior (12, 47). Although our findings here are consistent with a role of *Toxoplasma* in the human brain, it is of note that this study was performed following infection with tachyzoites. Since the majority of people infected with the parasite are in the chronic stage of infection, in which the parasite is largely in cyst form, the extent to which our findings will generalize to the chronic stage of infection is not known. Studies from tissues infected with cysts would be useful in order to further define the effects of *Toxoplasma* infection on human neural cells.

(ii) Modulation of metabolism. The metabolism pathway analysis showed that cells infected by the type I strain downregulated gene transcripts involved in synthesizing carbohydrates, lipids, and amino acids (Table 3; see also Data Set S6 in the supplemental material). Our findings are similar to those of Knight et al. (30), who reported that many gene transcripts, including those for ribosomal proteins, transcription factors, and certain mitochondrial proteins, were suppressed by type I strain infection at 24 h postinfection. An explanation for this scenario is that the inhibition of genes involved in host cell metabolism is a consequence of stress response. It is possible that the disruptive effects (1,122 differentially expressed RefSeq genes) imparted by type I infection took over the host cell and thus compromised the host metabolic machinery.

(iii) Modulation of signaling. The signaling pathway analysis in type I strain-infected cells showed that the inhibition of these pathways limits inflammation (Table 3; see also Data Set S6 in the supplemental material). Interleukin 8 is a proinflammatory cytokine; downregulation of genes involved in IL-8 signaling suggests a disability of the immune system to generate a proinflammatory response via the IL-8 signaling pathway. HMGB1 is a chromatin binding protein known to be a potent proinflammatory stimulus through binding of IL-1, TNF-α, and IFN-γ to their own receptors (11). It has been reported that IFN-γ-induced cells infected with an avirulent strain of *T. gondii* will release HMGB1 during necrosis to stimulate local immunity, but infections with virulent strains do not result in this release (60). Consistently, we found an inhibition of HMGB1 signaling in type I strain-infected cells, which suggested a potential inhibitory effect on HMGB1-mediated proinflammatory responses. Moreover, inhibition of the insulin-like growth factor 1 (IGF-1) signaling pathway has been suggested to play a role in decreased immune cell survival (39). It is likely that the host cells did not mount a strong response directed at alerting and activating the immune system to react to the infection. This observation was in agreement with previous reports which found that *Toxoplasma* type I manipulates host signaling cascades through a general strategy to control proinflammatory responses (7). Robben et al. (42) also reported that only very low levels of proinflammatory cytokines have been induced in type I strain-infected mice. It is of note that in the current study, cholecystokinin (CCK)/gastrin-mediated signaling was strongly altered by type I infection of neural cells. CCK is the most abundant peptide system in the brain, acting as a satiety promoter to regulate food intake (25). Recent studies have documented that type I and III strains activate STAT3 and STAT6 via the rhoptry kinase ROP16 (44). Although SK-N-MC cells could express STAT3 (46), we did not observe any gene changes on the STAT pathway, e.g., JAK. An explanation for this is that various host cells have differing responses to *Toxoplasma*, and it is possible that *Toxoplasma* has an alternative method of reducing these STAT levels in SK-N-MC cells.

(iv) Gene networks. FOS, IFN-γ, and ERK1/2 have a central role in the merged signaling network describing molecular communications due to type I strain infection (Fig. 5). The *FOS* gene encodes one of the subunits comprising the transcription factor complex AP-1, which is involved in cell growth, survival, and differentiation. Highly significantly upregulated FOS as well as EGR (early growth response) (see Data Set S1 in the supplemental material) may represent an activation of host defenses to infection. Given the well-documented roles that the EGR and AP-1 transcription factors play in stress responses (10, 38), the activation of these stress response transcription factors may be important to help host cells survive the stress of the infection. Among cytokines, IFN-γ is the most important for resistance to both acute and chronic *Toxoplasma* infections. However, *Toxoplasma* has developed a means of immune evasion by subverting the IFN-γ signaling of the human host, regardless of which *Toxoplasma* strain is used (29). Consistent with this finding, our results showed no obvious alteration of IFN-γ by the type I strain or by the other 2 strains. ERK1/2 is a member of the mitogen-activated protein kinase (MAPK) family. Interference with MAPK signaling is believed to be an additional mode by which the type I strain prevents proinflammatory cytokine production during intracellular infection (28). Since IFN-γ and MAPK signal transductions are important targets of manipulation by the parasite (1), suppression of these molecules suggests downmodulated immune responses.

Significant transcript changes specific to type III infection. (i) Modulation of metabolism. The GO analysis showed a marked effect on nucleotide metabolism, including ribosome biogenesis and ncRNA metabolism and processing in type III-infected cells (Fig. 4). It has been proposed that yeast cells

regulate ribosome biogenesis primarily in response to growth-limiting environmental signals (33). Stress of infection may therefore represent such signals that can activate signaling pathways to upregulate the expression of ribosome biogenesis. Gene transcripts that synthesize amino acids and lipids were also generally upregulated (Table 3; see also Data Set S6 in the supplemental material). Overall, these data indicate that cells rapidly respond to *Toxoplasma* type III infection by upregulating many gene transcripts involved in the metabolic process, perhaps to compensate for nutrient scavenging caused by intracellular organisms.

(ii) Modulation of signaling. CD27 and PKR were the signaling pathways most affected by type III strain infection (Table 3). CD27, a member of the tumor necrosis factor receptor (TNFR) superfamily, acts in concert with the T cell receptor to support T cell expansion (21). More specifically, CD27 makes its contribution through promoting survival of activated T cells and complements CD28 (20). Therefore, the alteration of genes involved in CD27 signaling indicates an effective host immune response. PKR is known to have antiviral properties due to its role in translational control. However, a possible role of PKR in protozoan parasitic infection (*Leishmania*) has been reported recently (41). The observation of an association between *Toxoplasma* infection and PKR has not been reported previously.

(iii) Gene networks. The merged results from the 4 top signaling-related networks due to type III strain infection indicate that ERK1/2 and TNF have a central role (see Fig. S2 in the supplemental material). No obvious alteration of ERK1/2 was similar to what we observed following infection with the type I strain. Recently, it has been reported that TNF-α activity is strongly suppressed by both virulent and avirulent *Toxoplasma* strains (32). Consistent with this finding, our results showed a suppression of TNF-α by the type III strain as well as by the other 2 strains.

Significant transcript changes specific to type II infection. (i) Modulation of metabolism. No significant effects on metabolism pathways of cells infected with the type II strain were observed (see Data Set S6 in the supplemental material). An explanation for this is that type II infection has a subtle effect on the host, as only a small group of genes (78) were altered and these were related to circadian rhythm, growth, and signaling. Blader et al. (5) have reported an upregulation in host transcripts encoding glycolytic and mevalonate enzymes from human foreskin fibroblast cells infected by type II strains at an MOI of 5 to 10. In addition to the difference in cell types examined, the substantial difference in MOIs (3 compared to 5 to 10) may play a role in the different findings following type II strain infection. Robben et al. (42) reported that the levels of IL-12 induction caused by type II strain infection were related to the infectious dose; hence, higher MOIs could cause greater changes that would stimulate host defense for survival, including upregulation of some metabolic transcripts.

(ii) Modulation of signaling. Prolactin signaling was the pathway most affected by type II strain infection (Table 3). The most important roles of prolactin, aside from reproduction, are played in water-electrolyte homeostasis, influence on brain and behavior, regulation of growth and development, and immunoregulation (6). Elevated prolactin levels have been found in patients with schizophrenia following antipsychotic treatment (36). Prolactin secretion in humans is increased by physical or emotional stress (40) and is associated with sleep-related rhythms (45). Consistently, the circadian sleep cycle was one of the biological processes most affected by type II strains in GO analyses (Table 2). Moreover, a protective action of prolactin during *Toxoplasma* infection in mice was reported previously (3).

(iii) Gene networks. The signaling-related network due to type II strain infection indicates that ERK1/2 and TNF have a central role (see Fig. S1 in the supplemental material). As for the other strains, the suppression of these molecules is likely to result in a suppression of the host immune response.

Conclusion. According to previous reports and the data presented herein, it is clear that the different canonical *Toxoplasma* strains elicit large differences in gene expression levels in infected cells. These differences are both quantitative and qualitative and involve a wide range of biological functions. It is particularly striking that infection with *Toxoplasma* type I led to changes in the expression levels of many genes involved in brain development and transmission of nerve impulse, while infection with *Toxoplasma* type III led to changes in ncRNA. Although infection with *Toxoplasma* type II yielded a small number of gene changes, some altered biological processes that related to hormones such as prolactin and insulin are of importance. Both canonical pathway and network analyses suggest that the three *Toxoplasma* strains employ somewhat different strategies to avoid, deflect, or subvert host defense mechanisms. These differences may explain some of the differences in terms of clinical consequences of *Toxoplasma* infection in infected individuals.

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